Identification of an immunodominant epitope in the C terminus of glycoprotein 5 of porcine reproductive and respiratory syndrome virus

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Glycoprotein 5 (GP₅) is the major glycoprotein of porcine reproductive and respiratory syndrome virus (PRRSV). Expression of GP₅ has been improved by removing the transmembrane regions. Vectors were constructed encoding complete GP₅ plus three mutants: GP₅ΔNs (residues 28–201), GP₅[30–67] (residues 30–67) and GP₅[30–201] (residues 30–67/130–201). The three deletion mutants were expressed at levels 20–30 times higher than complete GP₅. GP₅[30–201] was well recognized in ELISA or immunoblotting by a collection of pig sera. All the fragments were tested for the generation of MAbs, but only the polyhistidine-tagged fragment GP₅[30–201]H elicited an antibody response sufficient to produce MAbs. The two MAbs were positive for PRRSV in ELISA and immunoblotting, but negative for virus neutralization. MAb 4BE12 reacted with residues 130–170 and MAb 3AH9 recognized residues 170–201. This region was recognized strongly in immunoblotting by a collection of infected-pig sera. These results indicate diagnostic potential for this epitope.

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of a reproductive disorder and respiratory problems in pigs (Wensvoort et al., 1991; Benfield et al., 1992; Morrison et al., 1992). PRRSV is a spherical, enveloped RNA virus, with particles 45–70 nm in diameter, that belongs to the genus Arterivirus (Conzelmann et al., 1993; Meulenberg et al., 1993). The virion is formed by three major structural proteins, a 15 kDa nucleocapsid (N) protein, an 18 kDa membrane (M) protein and the 25 kDa envelope-associated glycoprotein 5 (GP₅) (Meulenberg et al., 1995).

GP₅ contains most of the epitopes involved in virus neutralization (Pirzadeh & Dea, 1997). Previous studies have reported the production of neutralizing MAbs specific for PRRSV GP₅ (Pirzadeh & Dea, 1997; Wieczorek-Krohmer et al., 1996; Zhang et al., 1998) by using recombinant proteins or pre-induction of tolerance against non-infected porcine alveolar macrophages. Also, pigs vaccinated with plasmid DNA encoding GP₅ (Pirzadeh & Dea, 1998) or baculovirus-expressed GP₅ plus GP₂ (Plana Duran et al., 1997) were partially protected.

From a structural point of view, GP₅ contains two N-glycosylation sites that are highly conserved among strains from Europe and North America and a very hydrophobic region in the N terminus, which could act as a leader sequence. GP₅ also contains several hydrophobic transmembrane regions. These hydrophobic domains probably provoke retention of the protein in the ER, causing inhibition of the synthesis of GP₅.

Fig. 1. Schematic representation of PRRSV GP₅ fragments. The fragments were constructed as described in Methods by using PCR on the original cDNA clone. The first and the last GP₅ residue are indicated above each fragment. Fragment names are indicated on the right. Primers used in PCR are indicated by single letters and primer sequences are shown below.

A: 5’-GCTGTGGATCGGCATGTCC-3’
B: 5’-TGGAAAGATGTCCTAGGC-3’
C: 5’-CTGATCATGTGTCCTGGCGGACAGCTCG-3’
D: 5’-GGCAAGCCCATGGAAATTATGACCTCCACACCATAATG-3’
E: 5’-TTTATTGGGATACCAAAATTACGTGCT-3’
F: 5’-GATGTCAGAGGAGGATCCACACTCATCGAC-3’
G: 5’-AAATTGGCAAGGATCAGTCGGTGCGAC-3’
H: 5’-CGCACAGGAATCTAGGC-3’
in expression systems such as baculovirus (Plana Duran et al., 1997). However, removal of the transmembrane region has improved considerably the solubility of similar proteins such as gp55 of classical swine fever virus (Hulst et al., 1993). A similar approach was also used for epitope mapping of the Gp protein of equine arteritis virus (EAV) (Chirnside et al., 1995).

In general, proteins containing hydrophobic or membrane-associated domains are insoluble and difficult to recover. In order to study whether deletion of these anchoring hydrophobic regions could enhance expression of PRRSV GP₃, we tested the expression of several deletion mutants of GP₃ in E. coli. The same fragments were used to immunize mice in order to produce MAbs, which were then used to define an important antigenic domain in GP₃.

Transmembrane regions in the amino acid sequence of GP₃ were identified by using the program PredictProtein (EMBL) (Rost, 1996; Rost et al., 1995). There are two major transmembrane domains, regions 14–31 and 64–134, of a total of 201 residues. Based on these data, we prepared three GP₃ mutants: GP₃ΔNs, spanning residues 28–201, which has the signal peptide deleted, and GP₃[30–67] (residues 30–67) and GP₃[30–201] (residues 30–67 plus 130–201), which have respectively two and one transmembrane region deleted (Fig. 1).

Plasmid pPRRS-ORF5 (Plana Duran et al., 1997) was used as the starting material for subcloning. This plasmid contains ORF5 of PRRSV Olot strain (GenBank accession no. X92942). For expression of complete GP₃, pPRRS-ORF5 was digested with BamHI and subcloned into the BamHI site of pET3Xb (Studier et al., 1990). The primers used in construction of the deletion mutants are shown in Fig. 1. All PCR amplifications were carried out with 50 ng template DNA, 800 ng primers and Vent DNA polymerase (Biolabs). The PCR product was digested with Sau3AI and inserted into the BamHI site of pET3Xc. To prepare the deletion mutants GP₃[30–67] and GP₃[30–201], we followed a procedure based on a PCR with a chimeric intermediate oligonucleotide (Pont-Kingdon, 1994). The PCR conditions consisted of five cycles at a permissive hybridization temperature of 42 °C and 25 cycles at a restrictive hybridization temperature of 60 °C. Three products, of 520, 360 and 111 bp, were obtained after PCR amplification. The PCR fragments of 360 and 111 bp were cloned into pET3Xc. After verification of the sequence, competent cells of E. coli strain BL21(DE3)-pLysS were transformed with each of the recombinant pET3X-derived plasmids and expression was induced with 0.4 mM IPTG. Recombinant proteins were collected and purified as described previously (Martinez-Torrecuadrada & Casal, 1995).

Briefly, 20 ml cell cultures were harvested 3 h after induction by centrifugation at 4000 r.p.m. for 5 min, washed twice with PBS and analysed on an 11% SDS–polyacrylamide gel. Complete GP₃ was expressed at low levels (0.08 mg/ml), as expected from its structural properties and its toxicity for cells. The remaining fragments, ΔNs, 30–67 and 30–201, yielded recombinant truncated fusion proteins of the expected sizes (51–2, 36–8 and 45–2 kDa), including the 260 amino acids of the gene 10 leader peptide of phage T7 expressed as the fused protein. They were expressed at much higher levels (1.5, 1.6 and 2.6 mg/ml). The identity of the proteins was confirmed by immunoblotting with PRRSV-specific pig antisera. Recombinant proteins were partially purified by solubilization of inclusion bodies with Triton X-100 and 4 M guanidine chloride. The purity of the proteins recovered was around 60–70%.

In order to study the antigenicity of the different GP₃ fragments and to learn whether the different domains of GP₃ were recognized equally well by sera from PRRSV-infected pigs, the reactivity of the different fragments was analysed by immunoblotting. We used a collection of 13 European, two Canadian and two PRRSV-negative pig sera. Complete GP₃ was recognized weakly, probably because of its lower level of expression. However, all but one of the sera reacted well with fragments GP₃ΔNs and GP₃[30–201]. In contrast, fragment GP₃[30–67] was recognized by only two pig sera. Reactivity was isolate-specific, as none of the Canadian pig sera recognized the European GP₃ΔNs mutants, and was focused on the C-terminal part of GP₃, residues 130–201.

To determine the immunogenicity of the recombinant truncated fragments of GP₃ in mice, partially purified PRRSV GP₃ and fragments were used for immunization of BALB/c mice. Each mouse received three doses of 50 µg protein in Freund’s adjuvant at 15-day intervals and mice were bled for serological analysis 7 days after the last immunization. Complete GP₃ and fragment GP₃ΔNs were less immunogenic, as respectively no mice or only one mouse elicited antibodies that reacted against the virus in ELISA or immunoblotting. In the case of GP₃[30–201], antibodies from three of four mice were able to recognize the virus. GP₃[30–67] showed intermediate immunogenicity; two of four mice developed PRRSV-specific antibodies. The four recombinant proteins were tested for the production of MAbs, without success. Several factors could explain these poor results: low immunogenicity of PRRSV proteins in mice, insufficient purification of the recombinant proteins or interference of the fused protein. In fact, there is only one previous report (Wiczkorek-Krohmer et al., 1996) describing the production of MAbs against a European PRRSV GP₃.

In order to determine whether the purity of the proteins influenced these results, a new fragment, GP₃[30–201]H₃, was subcloned in pET28c (Novagen). This construct allowed expression of fragment GP₃[30–201] with a polyhistidine tail at the N terminus. GP₃[30–201]H₃ (17.1 kDa) was expressed at an intermediate level and reacted well with the pig antisera by immunoblotting. The fragment was further purified by using Ni²⁺-NTA columns (Qiagen). Briefly, the recombinant material was solubilized in TSG buffer (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine–HCl) and clarified by centrifugation. Elution of GP₃[30–201]H₃ was
achieved with 200 mM imidazole in TSG buffer. The recovery of final product, after purification, was 3 mg/l with 90% purity. This highly purified fragment was much more efficient in immunization of mice. All immunized mice developed high ELISA titres against PRRSV virions (> 1/8000). Thus, it was selected for the production of MAbs.

Protocols for immunization and the preparation of MAbs have been described previously (Rodriguez et al., 1997). After immunization of mice with GP₅[30–201]H, two MAbs specific for PRRSV GP₅ by ELISA were produced. The isotypes of the MAbs were IgG2b for 3AH9 and IgG2a for 4BE12. The protein specificity of the two MAbs was confirmed by immunoblotting analysis. Both MAbs reacted with the 25 kDa band corresponding to viral GP₅ (Fig. 2). Also, the MAbs recognized the native virus protein by indirect immunofluorescence and immunoperoxidase assays, suggesting that they recognized linear epitopes. In contrast, no reactivity was detected with North American strains of PRRSV by ELISA or immunoblotting (data not shown).

Neither the two MAbs nor the mouse sera neutralized PRRSV. In contrast, pig sera gave neutralization titres between 1/2 and 1/625. These results agree with those of Pirzadeh et al. (1998), who also found no neutralizing activity in hyperimmune sera from mice immunized with GP₅ from five different isolates (Pirzadeh et al., 1998). Since the proteins were expressed in E. coli, we could speculate that this lack of neutralization resulted from incorrect folding of the proteins. However, attempts to express the same fragments in baculovirus were unsuccessful.

The antigenic domain recognized by the MAbs in PRRSV GP₅ was identified by probing the reactivity of the two MAbs with the four truncated GP₅ fragments. The MAbs reacted with fragments ΔNs and GP₅[30–201], but not with GP₅[30–67] (Fig. 2). For fine mapping of MAbs 3AH9 and 4BE12, the C-terminal region (aa 130–201) was split in two fragments, as shown in Fig. 1. Fragments were prepared by PCR with the indicated primers (Fig. 1). Fragment 130–170 was cloned in pET3Xa and fragment 170–201 was cloned in pET3Xb. The fragments were expressed and transferred to Immobilon-P membranes for immunoblotting analysis. The results are shown in Fig. 3(A, B). The two MAbs recognized different epitopes, one in each fragment. MAb 4BE12 reacted with fragment 130–170 and 3AH9 reacted with fragment 170–201, as well as with the virus and fragment 30–201.

In order to determine whether one of the two regions was recognized predominantly in natural infections, the reactivity of a collection of pig sera from infected animals was tested with the two small C-terminal fragments by immunoblotting. All the pig sera recognized fragment 170–201 (Fig. 3D). In contrast, none of the sera reacted with fragment 130–170 or the control (Fig. 3C). This result indicates the immunodominance of epitope 170–201 in pigs. This sequence could have interesting applications in diagnostics, alone or in combination with the N protein, to facilitate earlier diagnosis of PRRSV, since antibodies directed against GP₅ are detected as soon as 7 days post-infection, whereas antibodies against the N protein are not detected until day 10 post-infection (Loomba et al., 1996).

An alignment analysis showed that region 170–201 is highly conserved in all European isolates and shows 60% identity to American isolates in the last 25 residues. This reinforces the significance of this epitope as a diagnostic tool. A potentially antigenic region was identified close to the C terminus on the basis of theoretical predictions (Goldberg et al., 2000). Interestingly, some predictions assigned fragment 170–190 as a transmembrane region (Dea et al., 2000). This prediction, together with our results, might suggest that only the last 11 residues (190–201) are accessible to antibodies on the surface of the virus.

Previous expression of PRRSV GP₅ in the baculovirus system was inefficient (Plana Duran et al., 1997; Kreutz & Mengeling, 1997), making the preparation of large amounts of the protein for characterization and immunogenicity studies difficult. Removal of the predicted transmembrane region has resulted in a significant improvement in expression levels, probably by redirecting the protein to inclusion bodies, from where they can be recovered by regular solubilization procedures. Subsequently, we have used these fragments to characterize the antigenic structure of GP₅. The definition of antigenic regions of GP₅ is very important for vaccine development and diagnostic purposes.

Our results indicate that, despite good antigenicity, the C-terminal region may not actually be involved in virus neutralization. Thus, either the hypervariable ectodomain of the protein is the region involved in virus neutralization or the neutralizing epitopes are mainly conformational. Considering that neutralizing epitopes were defined in the ectodomain for
Fig. 3. Identification of PRRSV GP₃ sequences recognized by MAbs 3AH9 and 4BE12 and reactivity with pig sera. (A)–(B) For fine mapping of the epitopes, MAbs 3AH9 (A) and 4BE12 (B) were made to react with fragments GP₃[130–170] and GP₃[170–201]. PRRSV virions and GP₃[30–201] were used as positive controls. Bound antibody was detected as described in Fig. 2. MAbs were incubated with lysates from E. coli cells expressing the T7 tag (lanes 1), GP₃[30–201] (2), GP₃[130–170] (3) or GP₃[170–201] (4) or with PRRSV virions (lane 5). The amino acid sequences recognized by each MAb are indicated below. (C)–(D) In order to analyse the reactivity of pig sera with epitope 170–C terminus by immunoblotting, cell lysates expressing only the T7 tag (C) or GP₃[170–201] (D) were prepared and separated on an 11% SDS–polyacrylamide gel. Proteins were transferred to PVDF membranes and the filters were incubated with a panel of PRRSV-positive pig sera and MAb 3AH9 as a positive control. Pig antibodies were detected with peroxidase-labelled Protein A (dilution 1:1000). The position of the recombinant fragment is indicated by an arrow. Positions of molecular mass markers are indicated in kDa on the left.

EAV G₁ (Balasuriya et al., 1997), it is easy to speculate that something similar might occur in PRRSV. However, preliminary results obtained after expressing a fragment containing the first 40 residues of GP₃ indicate that a much larger region is necessary to form the epitope. In that case, if the neutralizing epitopes require several sequences from different regions, it would make production of a subunit vaccine based on GP₃ very difficult. In any case, the immunogenicity of these GP₃ mutants should be tested in pigs to provide a definitive assessment of the relevance of these epitopes in virus neutralization.

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References


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